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(54) **Vaccine against non-A, non-B hepatitis, and method of preparation.**

(57) Non-A, non-B hepatitis associated antigen is purified, inactivated and may be utilized as a vaccine.

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TITLE MODIFIED
see front page

TITLE: DETECTION OF NON-A, NON-B HEPATITIS ASSOCIATED ANTIGEN

INTRODUCTION

The present invention is concerned with the discovery of the existence of a non-A, non-B hepatitis associated antigen and this invention is also concerned with the use of this antigen to identify infectious blood donors and to prepare a vaccine. It is realized that in the time span after discovery of the existence by the present inventors, there appeared an article by Shirachi et al, "Hepatitis 'C' Antigen in Non-A, Non-B Post-Transfusion Hepatitis," The Lancet, October 21, 1978. pages 853-856.

In recent studies non-A, non-B hepatitis has been found to occur in 10% of transfused patients in the United States, resulting in about 200,000 cases per year. Fatalities from non-A, non-B hepatitis in the United States probably number round 1,000 per year among transfusion-related cases.

PRIOR ART STATEMENT

Shirachi et al, The Lancet, October 21, 1978, pages 853-856.

Tabor et al, Viral Hepatitis, eds. G.N. Vyas et al, The Franklin Institute Press, Philadelphia, 1978, pages 419-421.

Tabor et al, The Lancet, March 4, 1978, pages 463-466.

Tabor et al, Gastroenterology, 76:680-684, 1979.

Gocke et al, The Journal of Immunology, 104(4): 1031-1032, April 1970.

THE NON-A, NON-B ANTIGEN AND ITS ANTIBODY

Many cases of acute and chronic hepatitis which do not result from infection by either hepatitis A virus (HAV) or hepatitis B virus (HBV), are called "non-A, non-B hepatitis," and now account for 89% of cases of post-transfusion hepatitis in the United States. The presence of a transmissible agent in this disease has been demonstrated by its transmission to chimpanzees by the inoculation of serum from humans chronically infected with non-A, non-B hepatitis, and by serial passage to additional chimpanzees. Recently an antigen-

10. The method of any one of claims 1 through 9 wherein the antigen is inactivated by at least one of:

- heat treatment; or
- treatment with formalin.

11. A method of preparation for a vaccine effective against non-A, non-B hepatitis infection in mammals comprising:

- isolating the antigen associated with non-A, non-B hepatitis from specimens of blood serum, tissue, or a cell culture, taken from a donor mammal known to be infected by non-A, non-B hepatitis;
- preliminarily purifying the antigen;
- separating immunologically active polypeptides from the antigen by:
- detergent treatment,
- limited hydrolysis, or
- reduction; and
- inactivating said immunologically active polypeptide.

12. The method of claim 11 wherein an immunologically active polypeptide is purified before being inactivated by the technique of:

- fractional precipitation;
- solubilization;
- gel filtration;
- molecular sieving;
- affinity, adsorption, or ion-exchange chromatography;
- density gradient centrifugation;
- electrophoresis; or
- countercurrent distribution.

13. The method of claim 11 wherein an immunologically active polypeptide is further purified by at least one of:

- alterations in pH;
- chemical treatments; and
- enzyme treatments.

14. The method of any one of claims 11 through 13 wherein an immunologically active polypeptide is inactivated by at least one of:

- heat treatment; or
- treatment with formalin.

Sera stored at -20°C were tested by CEP using 1% agarose (Indubiose A37, L'industrie Biologique Francaise, Gennevilliers, France) in barbital buffer, pH 8.6, poured onto 3.5 x 12.5 cm glass plates (16 ml per plate). Melted agarose (16 ml) was poured onto a lantern slide. When it had cooled, two rows of holes were punched into the agarose. Antibody was placed in one row of holes and samples to be tested were added to the other row. When testing for antibody, antigen was added to one row and samples in the other row. The lantern slide was placed in a CEP chamber. Paper wicks were used to connect each side of the slide to each of two pools of barbital buffer, pH 8.6. An electric current was passed across the plate, 35 milliamps per plate, for one hour. Immunoprecipitin lines were read after 1, 24, and 48 hours of storage in a moist chamber at room temperature. When the test sample was positive, a precipitin line was seen between the rows, using the naked eye with the aid of an electric lamp.

RADIOIMMUNOASSAY (RIA)

Antibody to the non-A, non-B hepatitis was purified by precipitating it from serum using 30% ammonium sulfate. This purified antibody was labeled with radioactive iodine using the chloramine-T method. Unpurified antibody was coated on plastic beads. The coated beads

were placed in wells of a plastic plate. Samples to be tested for antigen were added to each well. After 18 hours incubation, the excess sample (other than any antigen which was then attached to the bead) was washed away. The radio-labeled purified antibody was then added to the wells and incubated for three hours; the excess was washed away. The amount of radioactivity adhering to the beads was counted in gamma counter. Positive results were identified by the detection of radioactivity on the beads, in comparison to negative samples. The presence of antibody was determined by adding the sample to be tested to a known antigen-positive serum, and then, following incubation for one hour, testing the mixture for antigen. The presence of antibody was identified by the decrease in radioactive counts compared to the result obtained using the antigen alone.

In addition to CEP and RIA used to detect antigen and antibody, alternate immunological methods may be used to detect the antigen including agar gel diffusion, passive hemagglutination, latex agglutination, complement fixation, and enzymes linked immuno-sorbent assay.

THE ANTIGEN

An abbreviated or capsulized description of purification

for the associated antigen and active subunits is summarized as follows.

The non-A, non-B hepatitis associated antigen was purified from serum (or tissue and cell cultures when the agent is propogated) by selection from the following techniques:

(1) Fractional (selective) precipitation or solubilization

(2) Gel filtration, molecular sieving

(3) Chromatographic techniques (affinity, adsorption or ion-exchange chromatography)

(4) Density gradient centrifugation

(5) Electrophoresis including isotachophoresis and isoelectric focusing

(6) Countercurrent distribution

Further purification treatments include alterations in pH, chemical treatments and enzyme treatments.

Subunits

Immunologically active subunits of the non-A, non-B hepatitis associated antigen have been prepared following preliminary purification of the antigen by a selection from the following:

(1) detergent treatment

(2) limited hydrolysis

(3) reduction

Immunologically active polypeptides have been separated here by procedures outlined above.

Development of In Vitro Tests

By inducing antibody specific for the non-A, non-B associated antigen in suitable animal species; i.e., chimpanzees, or selecting human sera containing these antibodies, immunologic tests to detect the antigen (such as Agar gel diffusion, counterelectrophoresis, complement fixation, passive hemagglutination, radio-immunoassay or enzyme-linked immuno-sorbent assay) have been developed and used to (1) detect persons transmitting non-A, non-B hepatitis and (2) identify sources of antigen for in vitro tests and vaccine production.

Vaccine

A direct use of purifed antigen or immunologically active subunits inactivated by either heat, formalin or both may be conventionally utilized as a vaccine.

The table below shows a summary of clinical testing.

TABLE 1

| Patients Tested | | Non-A, Non-B Antigen | Non-A, Non-B Antibody |
|---|---|---|---|
| 54 | Normal volunteer blood donors | 0 | Not tested |
| 3 | Humans with chronic, non-A, non-B hepatitis who transmitted the disease to humans and chimpanzees | 3 | |
| 31 | Blood donors who transmitted non-A, non-B hepatitis one to four years previously | 11 | 5 |
| 12 | Humans with non-A, non-B hepatitis (weekly samples) | 8 | Not tested |
| 2 | Humans who recoverd from non-A, non-B hepatitis | 0 | 2 |
| 152 | Hemophiliac patients | Not tested | 59 |

EXAMPLE 1

Serum samples were obtained from three humans with chronic non-A, non-B hepatitis. Blood from human #1 had caused non-A, non-B hepatitis in a nurse who accidentally cut herself on a piece of glass contaminated with his blood. Humans #2 and #3 had donated blood, and their blood had caused non-A, non-B hepatitis in recipients. Serum from all three (Humans #1, #2, and #3) was inoculated into chimpanzees and caused non-A, non-B hepatitis in the chimpanzees. The non-A, non-B hepatitis associated antigen was found in the blood of all three humans.

EXAMPLE 2

Serum samples were obtained from 31 blood donors whose blood had caused non-A, non-B hepatitis in patients who had been transfused with a single unit of their blood (and no other blood) one to four years previously. The non-A, non-B hepatitis associated antigen was detected in 11 of these donors.

EXAMPLE 3

Serum was tested from 54 normal blood donors. None had the non-A, non-B hepatitis associated antigen.

EXAMPLE 4

Five of the 31 implicated blood donors (confer Example 2) had antibody to the non-A, non-B associated antigen, but no detectable antigen. The antibody in these cases indicated the presence of a different state of disease and was also an indication that in some cases their blood would transmit the disease, as it had done previously.

EXAMPLE 5

Chimpanzee studies

Weekly serum samples from seven chimpanzees beginning four weeks before incoluation with human non-A, non-B hepatitis were tested. The inoculation and course of infection in these chimpanzees are described in the three Tabor et al articles noted in the Prior Art Statement, supra. Each chimpanzee was infected by intravenous inoculation of serum from one of these humans chronically infected with non-A, non-B hepatitis. Chimpanzees #922, #930, #911, #916, and #946 were infected by inoculation with Inoculum I, or with acute phase serum from a chimpanzee infected by Inoculum I (Inoculum I passage). Chimpanzee #918 was infected by Inoculum II and #919 by Inoculum III. A convalescent

serum from each chimpanzee was used as antibody in CEP against that chimpanzee's own weekly serum samples; in three chimpanzees (#922, #918, #919), the convalescent serum was obtained after two intravenous inoculations with infectious serum. In addition, convalescent serum from chimpanzee #922 was used to test all chimpanzee serum samples studied.

Results. The antigen was detected in the sera of six of seven chimpanzees during non-A, non-B hepatitis. In general, the antigen was detected during the time of elevated aminotransferase levels but without a strict correlation with histopathologic changes in liver biopsy specimens. Chimpanzee #922 (Inoculum I) had elevated aminotransferase levels from Week 2 to 16 and had antigen detectable at Weeks 4-9 and at Week 15. Chimpanzee #930 (Inoculum I) had elevated aminotransferase levels from Week 3 to 23 and had antigen detectable at Weeks 2-8 (including two serum samples shown to transmit non-A, non-B hepatitis to experimentally inoculated chimpanzees) and at Week 18. Chimpanzee #911 (Inoculum I passage) had elevated aminotransferase levels from Week 5 to 21 and had antigen detectable at Weeks 19 and 20. Chimpanzee #946 (Inoculum I passage) had elevated transferase levels from Week 3 to 11 and had antigen detectable at Weeks 9, 10, 12, and 16. Chimpanzee #918 (Inoculum II) had

elevated aminotransferase levels from Week 4 to 20 and had antigen detectable at Weeks 6, 11, 14, and 15. Chimpanzee #919 (Inoculum III) had elevated aninotransferase levels from Week 3 to 20 and had antigen detectable at Week 3. The antigen could not be detected in serum samples from Chimpanzee #916 (Inoculum I passage).

The antigen could not be detected in any of 35 pre-innoculation serum samples from these chimpanzees, nor could it be detected in 28 weekly bleedings from three chimpanzees during experimentally induced hepatitis A or in 66 weekly bleedings from three chimpanzees during experimentally induced hepatitis B.

Antibody was detected in convalescent serum samples from all seven chimpanzees. Antibody was detected in every serum sample from chimpanzee #922 beginning with Week 28 after inoculation, 13 weeks after the disappearance of antigen and the return of aminotransferase levels to near normal values. Antibody remained detectable until longer than 19 months after inoculation. Titrations performed on selected serum samples from chimpanzee #922 before and after a second intravenous exposure to a non-A, non-B hepatitis inoculum (Inoculum III), revealed a four-fold increase in antibody titer. Ammonium sulfate precipitation and DEAE cellulose chromatography revealed the antibody to be in the 7S (IgG) fraction.

EXAMPLE 6

Human serum used as antibody in CEP included convalescent serum from the nurse (Human #1) who had recovered from non-A, non-B hepatitis 4 years earlier after the needlestick exposure to Inoclum I and convalescent serum from a multiply-transfused hemodialysis patient with a history of non-A, non-B hepatitis (Human #2).

Results. The antibody was detected in convalescent serum from Human #1 and Human #2. Antibody was not detected in any of two serum samples from the patient with chronic non-A, non-B hepatitis whose serum became Inoculum I.

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CLAIMS

1. A vaccine effective against non-A, non-B hepatitis infection in mammals comprising an inactive antigen isolated from a specimen of blood serum, tissue, or a cell culture of a donor mammal known to be infected with non-A, non-B hepatitis.

2. A vaccine effective against non-A, non-B hepatitis infection in mammals comprising an inactive immunologically active polypeptide isolated from specimens of blood serum, tissue, or a cell culture of a donor mammal known to be infected with non-A, non-B hepatitis.

3. A vaccine according to claim 1 in which the antigen has been purified after isolation.

4. A vaccine according to claim 2 in which the immunologically active polypeptide has been purified after isolation.

5. A vaccine according to any one of claims 1 through 4 wherein the donor mammal is a chimpanzee.

6. A vaccine according to any one of claims 1 through 4 wherein the donor mammal is a human being.

7. A method of preparation for a vaccine effective against non-A, non-B hepatitis in mammals comprising:

- isolating the antigen associated with non-A, non-B hepatitis from specimens of blood seeum, tissue, or a cell culture, taken from a donor mammal known to be infected by non-A, non-B hepatitis; and
- inactivating said antigen.

8. The method of claim 7 wherein the antigen is purified before being inactivated by the techniques of:

- fractional precipitation;
- solubilization;
- gel filtration;
- molecular sieving;
- affinity, adsorption, or ion-exchange chromatography;
- density gradient centrifugation;
- electrophoresis; or
- countercurrent distribution.

9. The method of claim 21 wherein the antigen is further purified by at least one of:

- alterations in pH;
- chemical treatments; and
- enzyme treatments.

antibody system detected by counterelectrophoresis (CEP) was described in humans with post-transfusion non-A, non-B hepatitis (Shirachi, et al, supra). In the present invention is reported antigen which is detectable by CEP in the serum of chimpanzees during the acute phase of experimentally induced human non-A, non-B hepatitis, an antibody which appears during convalescence, and the detection of this antigen-antibody system in humans with non-A, non-B hepatitis. The activity of the antigen has been shown in counterelectrophoresis (CEP) as well as in a solid phase radioimmunoassay.

Additionally, human tests showed antigen activity up to 1-5 years after transfusion in the donor. The tests enable blood banks to identify blood donors whose blood may transmit non-A, non-B hepatitis to recipients and eliminate the use of their blood for transfusions. This results in a decrease in the incidence of this disease. The test is also used to diagnose non-A, non-B hepatitis in patients.

An antigen was detected by counterelectrophoresis in serum samples from six of seven chimpanzees during the acute phase of experimentally induced non-A, non-B hepatitis using antiserum from a chimpanzee convalescent from human non-A, non-B hepatitis. This antigen could not be detected prior to the transfusion in 35 pre-

inoculation serum samples from these chimpanzees, or in 94 weekly bleedings from three chimpanzees with hepatitis A and three chimpanzees with hepatitis B.

The antigen was also detected in each of two serum samples obtained from a human with chronic hepatitis whose blood had transmitted non-A, non-B hepatitis to a nurse by accidental needlestick and to chimpanzees by experimental inoculation. In addition, the antigen was detected in serum obtained retrospectively from 11 of 31 former blood donors whose blood had transmitted post-transfusion non-A, non-B hepatitis several years previously to recipients of a single unit of their blood.

Antibody to this antigen was detected in convalescent serum samples from all seven chimpanzees studied, in convalescent serum from the nurse infected by accidental needlestick, and in serum from a hemodialysis patient convalescent from non-A, non-B hepatitis.

## COUNTERELECTROPHORESIS

Counterelectrophoresis (CEP) which may be also described as immunoelectrosmophoresis (IEOP) or immunoelectro-diffusion (IED) or countercurrentelectrophoresis is utilized as follows.

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# EUROPEAN SEARCH REPORT

0128995
Application number

EP 84 10 0106

## DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int. Cl. ³) |
|---|---|---|---|
| | No relevant documents have been disclosed<br><br>----- | | A 61 K 39/29 |

TECHNICAL FIELDS SEARCHED (Int. Cl. ³)

A 61 K

The present search report has been drawn up for all claims

| Place of search | Date of completion of the search | Examiner |
|---|---|---|
| THE HAGUE | 12-09-1984 | REMPP G.L.E. |

CATEGORY OF CITED DOCUMENTS

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